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Acidification of macrophage and fibroblast endocytic vesicles *in vitro*

(proton pump/pinocytosis/subcellular fractionation/lysosome/endosome)

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ABSTRACT **We have used the pH-dependent fluorochrome fluorescein-dextran (FD) to study the acidification of prelysosomal vacuoles (endosomes) and lysosomes isolated from cultured macrophages and fibroblasts. FD was internalized by pinocytosis under conditions that allowed its selective localization in endosomes (1- to 5-min pulse) or in lysosomes (5-min pulse, 30-min chase). Fibroblasts were also exposed to FD at 20°C, at which temperature endosome–lysosome fusion is inhibited. Cells were homogenized and labeled organelles were separated by centrifugation in Percoll density gradients. The addition of ATP rapidly decreased the internal pH of both endosomes and lysosomes, as indicated by a decrease in fluorescence intensity. The pH gradient was dissipated by H^+ ionophores and ammonium chloride. Acidification was not affected by inhibitors of the mitochondrial F_1,F_0-ATPase or the Na^+, K, K^+-ATPase and did not require permeant anions, Na^+, or K^+. Of the inhibitors tested, only *N*-ethylmaleimide prevented the ATP-dependent acidification of both compartments. These findings provide direct support for the existence of an acidic prelysosomal compartment that may be acidified via the same type of H^+ pump believed to operate in lysosomes and secretory granules.**

Pinocytosis typically results in the accumulation and degradation of internalized macromolecules in secondary lysosomes (1). However, delivery to lysosomes requires the participation of at least two other classes of endocytic vacuoles. Pinocytic vesicles, often with clathrin-containing coats, form at the plasma membrane and constitute the primary endocytic compartment. These vesicles subsequently fuse with, or fuse together to form, a somewhat larger class of secondary endocytic vacuoles (0.2–1.0 μm in diameter), referred to here as endosomes (1, 2). Endosomes can be distinguished from lysosomes in several ways: (*i*) they have a relatively low buoyant density (3–8); (*ii*) they are labeled by pinocytic markers more rapidly than and prior to labeling of lysosomes (7, 9); (*iii*) internalized macromolecules exhibit only a transient residence in endosomes, whereas lysosomes are typically the final destination (9); and (*iv*) endosomes are relatively devoid of acid hydrolases (3, 6–8).

These differences notwithstanding, endosomes may be similar to secondary lysosomes in at least one significant characteristic, low internal pH. This has been suggested by recent studies in which receptor-bound ligands were coupled to fluorescein (whose fluorescence spectrum is a titratable function of pH) and found to be internalized into an acidic compartment prior to delivery to lysosomes (8, 10). This was the case also with Semliki Forest virus (SFV), which requires a pH of <6 for fusion and penetrates into the cytosol from prelysosomal vacuoles (11). Here, we have used isolated endosomes labeled with the pH-sensitive fluorochrome fluorescein-dextran (FD) (12, 13) to study more directly the acidification of prelysosomal vacuoles. Two points are made: (*i*) both isolated endosomes and lysosomes can lower their internal pH via an ATP-dependent process and (*ii*) these data, when integrated with previously published results based on intact cells, provide strong evidence that prelysosomal endosomes are an acidic compartment.

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MATERIALS AND METHODS

Cells and Cell Culture. The mouse macrophage cell line J774 was maintained in suspension and baby hamster kidney fibroblasts (BHK-21) were grown in monolayer as described (14, 15).

Cell Labeling with Endocytic Markers. Horseradish peroxidase (HRP) (type II; Sigma) or fluorescein isothiocyanate-conjugated dextran (FD) (Sigma; average M_r = 64,000; 0.01 mol/mol of glucose; 2 mg/ml in Dulbecco's modified Eagle's medium) was used to label endocytic compartments (see legend to Fig. 1). Uptake was stopped by diluting and washing [by centrifugation with cold Hepes/saline (HS: 120 mM NaCl/5 mM KCl/10 mM Hepes, pH 7.4)]. Endosomes were labeled with a 1- to 5-min pulse in FD or HRP and lysosomes with a 5-min pulse, followed by a 30-min chase in marker-free medium.

Macrophage Cell Fractionation. After washing with cold HS, cells were resuspended (2–10 × 10^7 cells per ml) in cold homogenization buffer (0.25 M sucrose/2 mM EDTA/10 mM Hepes, adjusted with NaOH or, for Na^+-free experiments, with KOH to pH 7.4) and were homogenized (≈90% disruption) in a stainless steel Dounce homogenizer (Kontes). The homogenate was centrifuged (750 × *g*; 10 min at 4°C) and the resulting supernatant was diluted with cold homogenization buffer and Percoll (Sigma; in 0.25 M sucrose) to a final concentration of 27% Percoll (ρ = 1.066 g/cm^3). This mixture was layered over 3.0 ml of 2.5 M sucrose and was centrifuged for 2 hr (4°C) at 20,000 × *g* by using a Beckman Ti 70 rotor. One-milliliter fractions were collected from the bottom of the tube.

Baby Hamster Kidney Cell Fractionation. BHK-21 cell monolayers were labeled either at 37°C (30 min) or at 20°C (2 hr) with 3.5 mg of FD per ml of RPMI 1640 containing 0.2% bovine serum albumin and 10 mM Hepes (pH 6.8). After extensive washing with cold HS, cells were lysed (16) and a crude microsomal fraction was centrifuged as above (11).

Enzyme Assays. The distribution of HRP activity in the gradients was assayed in phosphate-buffered saline (pH 5.0) containing 0.1% Triton X-100 (TX) (9). Lysosomal hydrolase activities were determined by using *p*-nitrophenyl-coupled substrates (17) in 50 mM Na acetate (pH 5.0) containing 0.1% TX.

Abbreviations: FD, fluorescein-dextran; HS, Hepes/saline (120 mM NaCl/5 mM KCl/10 mM Hepes, pH 7.4); HRP, horseradish peroxidase; TX, Triton X-100; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; SFV, Semliki Forest virus.

Fluorescence pH Measurements. Changes in pH were recorded as changes in FD fluorescence intensity, which decreases as a function of decreasing pH (between pH 4 and pH 8) (12). Emission (515 nm) and excitation (485 nm) wavelengths were chosen to minimize the contribution of light scattering. pH estimates were determined from the fluorescence intensity at 515 nm (emission) relative to that observed after the addition of nigericin in KCl buffer (which was assumed to equilibrate internal and external pH). Nigericin was preferred to TX (13) because the latter significantly altered the light scattering properties of both the endosome and lysosome fractions. A standard curve relating pH and fluorescence intensity was obtained as described (13). Because it was not possible to control routinely for any nonlatent FD, the pH values obtained must be regarded as provisional. pH estimates based on excitation wavelength ratios (495 nm/450 nm) (12) were less reliable due to the differential effects of light scattering in endosomes and lysosomes as well as the effect of high Percoll concentrations (as in the lysosome fraction) on the fluorescence spectrum of FD. All measurements were made at ambient temperature by using a Perkin–Elmer 512 double-beam spectrofluorometer.

Endosomes (0.25 ml; 0.9 mg of protein per ml) or lysosomes (0.25 ml; 1.3 mg of protein per ml) were diluted into 2.5 ml of buffer containing 10 mM Hepes (adjusted to pH 7.4 with LiOH or KOH), 5 mM $MgCl_2$, 2 mM EDTA, and 125 mM KCl or, instead of KCl, 85 mM K_2SO_4, 250 mM sucrose, or 100 mM NaCl/25 mM KCl. Additions of various agents were made in water, ethanol, or dimethyl sulfoxide in volumes that were <1% of that of the incubation medium. The solvents alone had no effect. The reagents used and their final concentrations were: ATP (Na^+ or Tris salt; adjusted to pH 7.2 before use), 5 mM; valinomycin, 2.7 μM; carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), 18 μM; carbonylcyanide *m*-chlorophenylhydrazone (CCCP), 18 μM; nigericin, 0.2 μM; NH_4Cl, 11 mM; TX, 0.036%; *N,N'*-dicyclohexylcarbodiimide (DCCD), 36 μM; sodium azide, 1.1 mM; sodium orthovanadate (Na_3VO_4), 96 μM; efrapeptin, 0.91 μg/ml; *N*-ethylmaleimide, 0.91 mM. Reagents were purchased from Sigma, with the following exceptions: ATP, Boehringer Mannheim; sodium orthovanadate and DCCD, Aldrich. Efrapeptin was a gift of R. L. Hamill (Eli Lilly).

RESULTS

To use FD for studying endosome and lysosome acidification, it was necessary to define conditions that allowed selective labeling and density gradient separation of these two compartments. These were established by using both FD and another well-characterized marker of fluid pinocytosis, HRP (1, 9).

After a 1- to 5-min incubation of J774 macrophages in HRP-containing medium, cells were washed, homogenized, and centrifuged in self-forming Percoll gradients. As shown in Fig. 1*A*, a single major peak of peroxidase was detected at ≈1.03 g/cm^3. This peak was well separated from the major peak of lysosomal enzyme activity, which sedimented at a much higher density (≈1.09 g/cm^3) (Fig. 1*B*). However, the HRP did cosediment with a small amount of lysosomal markers, ≈10–15% of the total recovered activity of each of the three acid hydrolases assayed (β-*N*-acetyl glucosaminidase, β-galactosidase, and β-glucuronidase). Similar results were obtained for BHK-21 fibroblasts, although, as shown previously (11), low-density lysosomal hydrolase activity was not observed.

In contrast, when cells were pulsed in HRP for 5 min, washed, and then chased in HRP-free medium for 30 min, most (>75%) of the peroxidase cosedimented with the major peak of lysosomal enzymes (Fig. 1*A*). Similarly, J774 cells exposed to FD for 5 min, chased for 30 min, and again incubated in FD for 5 min prior to harvest exhibited two well-resolved peaks of fluorescence at 1.03 g/cm^3 and 1.09 g/cm^3 (Fig. 1*C*). Accordingly, these two peaks were operationally defined as endosomes and lysosomes, respectively. Analogous results were obtained for BHK-21 cells (not shown).

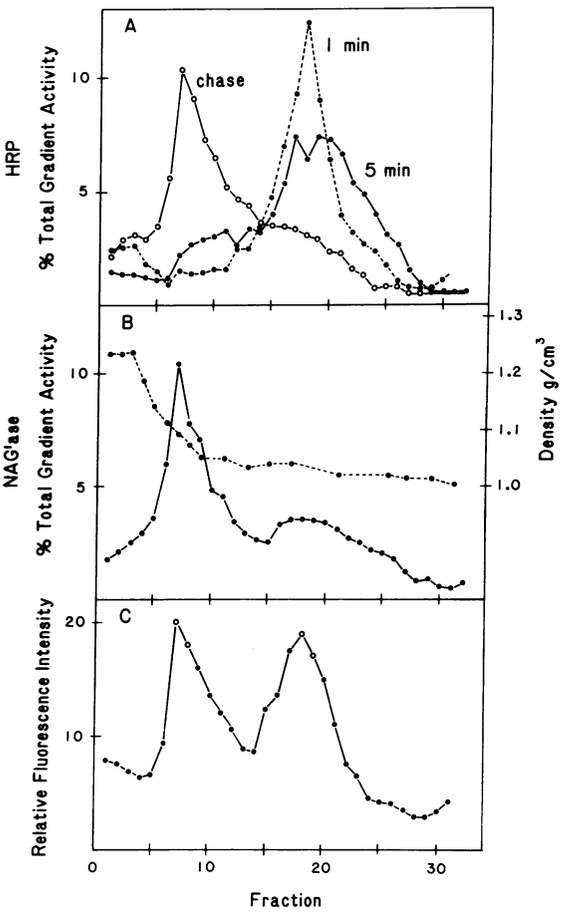

FIG. 1. Separation of endosomes and lysosomes by centrifugation in Percoll density gradients. (*A*) J774 (0.25–1.0 × 10^9) cells were labeled with HRP at 37°C for 1 min (●---●), 5 min (●——●), or 5 min and then maintained for 30 min in HRP-free medium (○——○) prior to homogenization. Total gradient activity was equivalent to 2.2 μg of HRP (1-min exposure) and ≈6.6 μg of HRP (5-min exposure); >75% of the HRP activity applied was recovered. The density of the endosome peak was 1.03 g/cm^3, whereas the lysosome peak was 1.09 g/cm^3 (see *B*). (*B*) The distribution of β-*N*-acetyl glucosaminidase (NAG'ase) activity (●——●) and the density of Percoll (●---●) in the gradients of *A* are shown. The major peak of enzyme activity occurs at 1.09 g/cm^3. Total activity recovered was equivalent to 25 μmol hydrolyzed per hr. (*C*) J774 cells were exposed to FD for 5 min at 37°C, maintained in FD-free medium for 30 min, and then maintained again in FD for the final 5 min prior to harvest and homogenization. The two peaks observed cosediment with the low-density (endosome) and high-density (lysosome) peaks of HRP activity. Open circles indicate the fractions used for acidification experiments.

Fluorescence Measurements of Isolated Organelles. When diluted either in sucrose or KCl buffer, endosomes exhibited a stable fluorescence with a pH close to that of the external buffer (Fig. 2). The addition of the K^+ ionophore valinomycin to the sample in KCl caused at best only a slight increase in fluorescence intensity (indicative of a slight internal alkalinization). Subsequent (or prior) addition of the H^+ ionophore FCCP caused a similar slight increase in fluorescence intensity (Fig. 2 *A* and *B*, upper traces). The effect of these two agents together could be mimicked by the addition of the ionophore nigericin, which catalyzes K^+–H^+ exchange (Fig. 2*C*, upper

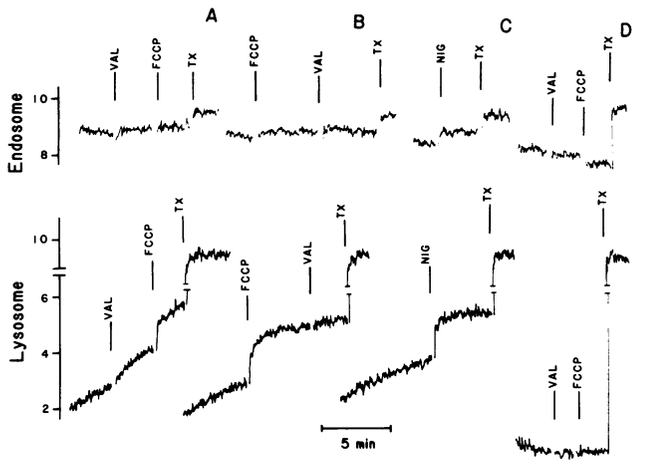

FIG. 2. Effects of ionophores on the fluorescence of FD-labeled endosomes (upper traces) and lysosomes (lower traces). Organelles in *A*, *B*, and *C* were diluted in (125 mM) KCl buffer; sucrose buffer was used in *D*. Relative intensity is given in arbitrary units. VAL, valinomycin; NIG, nigericin.

trace). However, in sucrose buffer valinomycin and FCCP caused a decrease in fluorescence intensity (indicative of internal acidification) (Fig. 2*D*, upper trace). Dilution into sucrose medium apparently creates a K^+ gradient (in > out) that serves as a driving force for acidification. In each case the addition of TX, which disrupted vacuole membranes and exposed the FD to buffer at pH 7.4, caused a further increase in fluorescence intensity.

FD-labeled J774 lysosomes diluted in sucrose buffer also exhibited a fluorescence (estimated at pH ≈ 6.7; see *Materials and Methods*) whose intensity remained constant over time (Fig. 2*D*, lower trace). However, in KCl buffer intensity increased gradually—as observed by Ohkuma *et al.* (13)—indicating an elevation of internal pH (Fig. 2*A*). Addition of valinomycin increased slightly the rate of drift, whereas FCCP caused a nearly immediate increase in intensity (Fig. 2 *B* and *C*). Presumably, the initial slow alkalinization was due to an exchange of external K^+ for internal H^+, which is hastened by the addition of valinomycin or FCCP. The more rapid effect of FCCP suggests that the resting permeability of the lysosome membrane is higher for K^+ than for H^+. Nigericin duplicated the effect of valinomycin with FCCP (Fig. 2*C*). However, in sucrose the absence of external cations prevented alkalinization by valinomycin or nigericin (Fig. 2*D*); however, NH_4Cl added to the external medium caused an increase in fluorescence due to influx and subsequent protonation of neutral NH_3 (not shown). Again, addition of TX in each case increased fluorescence intensity.

ATP-Dependent Acidification. Addition of ATP rapidly (within 30 sec) decreased FD fluorescence in both fractions (Fig. 3). Addition of a second aliquot of ATP did not result in any further decrease in fluorescence intensity (not shown). Endosome pH decreased ≈0.6 unit (pH ≈ 7.2 to 6.6); lysosome pH decreased ≈0.3 unit (pH ≈ 6.8 to 6.5). The extent of pH decrease in lysosomes (0.3–0.8 pH unit) was partly dependent on how long the lysosomes were incubated in KCl buffer prior to the addition of ATP (see above). Although the internal pH of isolated lysosomes was lower than that of endosomes, it was higher than that observed in intact J774 cells (pH 4.8–5.0; determined as in ref. 12). As expected, the ATP-generated fluorescence intensity decrease was reversed by FCCP (Fig. 3 *A–D*) or NH_4Cl (Fig. 3*E*). In contrast, the addition of valinomycin after acidification only slightly increased the internal pH, even when the organelles were diluted in KCl buffer (Fig. 3*D*); thus, the pH decrease was not generated by an electrochemical K^+ potential.

Substitution of sucrose buffer, devoid of permeant anions such as Cl^-, did not prevent acidification of either endosomes or lysosomes (Fig. 3 *C* and *E*). However, both the rate and extent of acidification were decreased, especially in lysosomes. Under these conditions, nigericin did not significantly elevate internal pH, because no external K^+ or Na^+ was available to exchange for internal H^+. However, NH_4Cl restored the internal pH to a value slightly higher than the initial one (Fig. 3*E*). In sucrose, reversal of acidification by FCCP occurred rapidly in endosomes and only slowly in lysosomes (Fig. 3*C*) [as observed by Okhuma *et al.* (13)]. Relative to KCl buffer, the rate or extent of acidification, or both, was decreased only slightly in K_2SO_4 buffer, in which the anion (SO_4^{2-}) was relatively impermeant (Fig. 3*B*).

ATPase Inhibitors. The effects of various inhibitors of known cellular ATPases were determined. Efrapeptin (Fig. 4*B*), NaN_3,

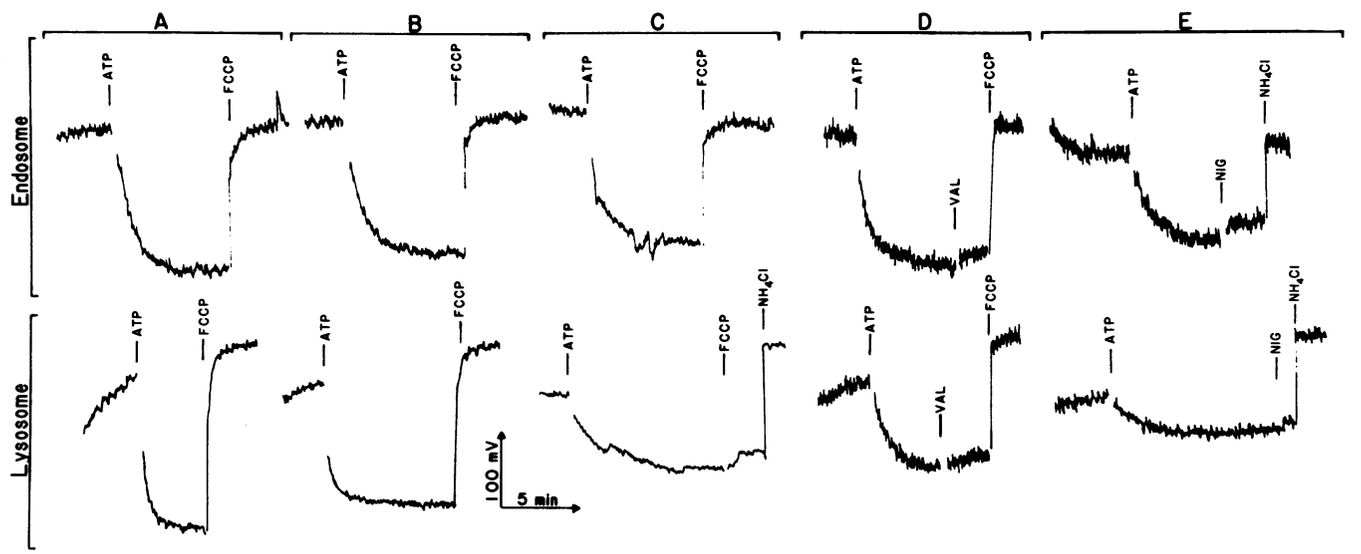

FIG. 3. ATP-dependent decrease in fluorescence in FD-labeled endosomes (upper traces) and lysosomes (lower traces). Organelles were diluted in isosmotic buffer containing 125 mM KCl (*A* and *D*), 85 mM K_2SO_4 (*B*), or 250 mM sucrose (devoid of permeant ions) (*C* and *E*). In each case, addition of 5 mM ATP caused a decrease in fluorescence intensity (indicative of a decrease in internal pH), which could be reversed by FCCP or NH_4Cl but not by valinomycin (VAL) (*D*). Nigericin (NIG) did not reverse the fluorescence decrease in the absence of external K^+ or Na^+ (*E*).

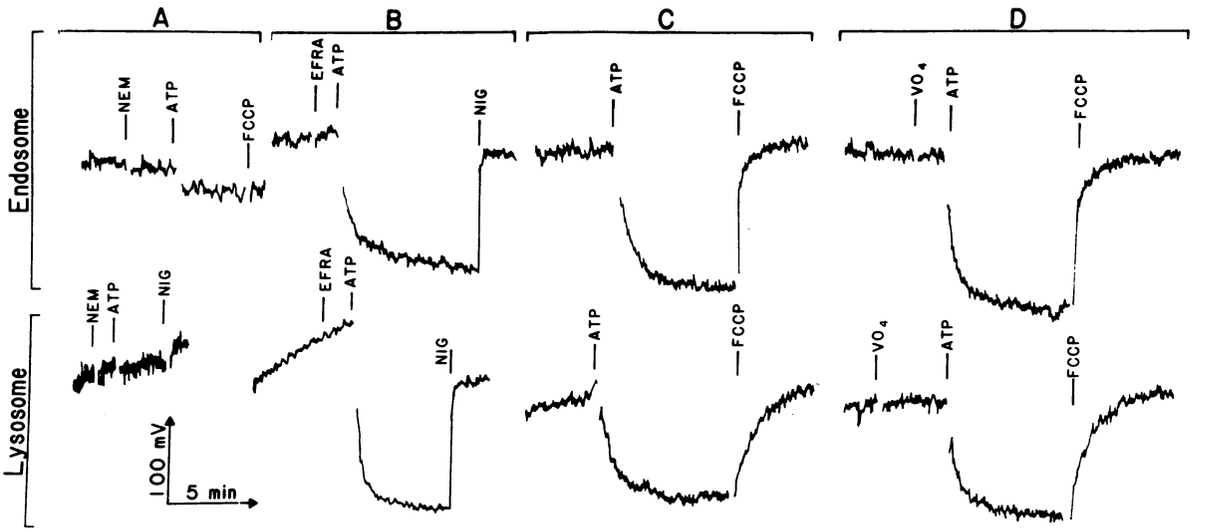

FIG. 4. Effects of ATPase inhibitors on ATP-dependent acidification of J774 endosomes (upper traces) and lysosomes (lower traces). Organelles were diluted in isosmotic buffer containing 125 mM KCl (*A* and *B*) or 100 mM NaCl with 25 mM KCl (*C* and *D*). In *A*, 0.91 mM *N*-ethylmaleimide (NEM) prevented the decrease in FD fluorescence after the addition of ATP. Efrapeptin (EFRA) (*B*) affected neither the rate nor extent of FD quenching when compared to controls (not shown). In *C* and *D*, ATP was added to endosomes and lysosomes under conditions (high [Na^+]) known to favor the activity of the Na^+,K^+-ATPase. However, the addition of vanadate (VO_4) had no effect on the ATP dependent fluorescence quenching in either fraction. NIG, nigericin.

or DCCD (not shown), at concentrations which inhibited the mitochondrial F_1,F_0-ATPase under the same assay conditions (not shown), did not affect the subsequent ATP-dependent decrease in FD fluorescence in either the endosome or lysosome fractions. However, high concentrations (100 μM) of DCCD were inhibitory, as reported for rat liver lysosomes (18). Similarly, sodium orthovanadate or ouabain (not shown) failed to inhibit acidification, even at high Na^+ concentrations which favor the activity of the Na^+,K^+-ATPase (Fig. 4 *C* and *D*). In control experiments the same concentration of vanadate inhibited >90% of the activity of purified Na^+,K^+-ATPase. Of the agents tested, only the sulfhydryl reagent *N*-ethylmaleimide caused a complete inhibition of ATP-dependent acidification in both endosomes and lysosomes (Fig. 4*A*).

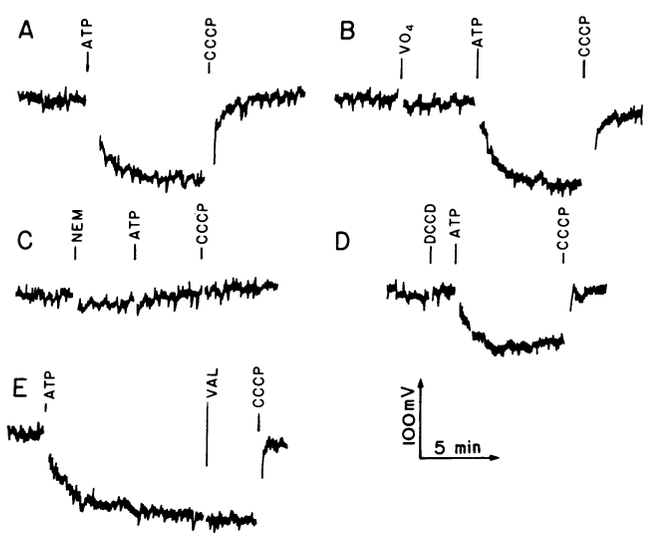

FIG. 5. Acidification of FD-labeled endosomes from BHK-21 fibroblasts. Endosomes were isolated from cells labeled with FD at 37°C (*A–D*) and diluted in isosmotic buffer containing 125 mM KCl (*A*, *C*, and *D*) or 100 mM NaCl with 25 mM KCl (*B*). ATP-dependent fluorescence quenching was reversed by CCCP (*A*, *B*, and *D*), nigericin, and NH_4Cl (not shown). Internal acidification was also observed in sucrose buffer (devoid of permeant ions) (not shown). Although 0.91 mM *N*-ethylmaleimide (NEM) inhibited completely acidification (*C*), neither vanadate (VO_4) (*B*) nor DCCD (*D*) had any effect. In *E*, a homogenate of BHK-21 cells, labeled with FD at 20°C, was diluted in KCl buffer prior to ATP addition. The decrease in fluorescence thus generated was reversed by CCCP but not valinomycin (VAL).

Acidification of Fibroblast Endosomes. Results obtained by using a FD-labeled endosome fraction from BHK-21 cells are shown in Fig. 5 and are virtually identical to those obtained for the J774 cell endosome and lysosome fractions. Acidification rapidly followed the addition of ATP, was not dependent upon external K^+, Na^+, or Cl^-, and was reversed by the H^+ ionophore CCCP (Fig. 5), nigericin, and NH_4Cl. Neither vanadate nor DCCD had any effect. However, *N*-ethylmaleimide inhibited completely the ATP-dependent acidification (Fig. 5*C*).

Experiments were also performed with homogenates of BHK-21 cells labeled with FD for 2 hr at 20°C. The transfer of endosome contents (e.g., SFV, FD, and HRP) to high-density secondary lysosomes is prevented at this temperature (11, 19). Fig. 5*E* demonstrates that endosomes labeled in this way also exhibit ATP-dependent acidification.

DISCUSSION

Traditionally, lysosomes have been viewed as unique among endocytic vacuoles due to their low internal pH. However, several recent reports have provided kinetic evidence that ligands internalized by receptor-mediated pinocytosis are delivered to an acidic compartment, prior to their appearance in hydrolytically active lysosomes (8, 10, 11). The results described in this paper not only support these observations but also suggest one mechanism for this acidification.

Our most important finding is that FD-labeled endosomes, as well as lysosomes, exhibit a decrease in pH (estimated to be 0.5–0.8 unit) upon the addition of ATP. The alterations in fluorescence were clearly due to intravacuolar acidification. Thus, NH_4Cl and H^+-conducting ionophores (FCCP, CCCP, and nigericin) prevented ATP-dependent fluorescence changes. These

data are in general agreement with those obtained by using partially purified fractions of rat liver lysosomes in conjunction with a variety of pH probes (e.g., FD, amino acid methyl esters, and [^{14}C]methylamine) (13, 18, 19). Thus, it is apparent that the membrane of endosomes contains an ATP-driven H^+ pump.

Interpretation of these data depends in part on the nature of the subcellular fractions used. Clearly, the endosomes and lysosomes employed represented crude preparations—i.e., they contained many other cellular components. However, these contaminants did not contribute to the fluorescence measurements because only endocytic vacuoles contained FD. More important is the fact that the procedures employed facilitated a clear separation of endosomes and lysosomes on the basis of both kinetic and density criteria (see the Introduction). It is not yet clear whether the small amount of lysosomal hydrolases found in endosome preparations (Fig. 1) (6, 7, 20) represent cosedimenting lysosomal or Golgi-derived vesicles or whether they are localized within the endosomes (i.e., FD-containing vacuoles) themselves. However, in recent work, we have separated the endosome and low-density hydrolase peaks by altering the conditions of centrifugation (unpublished data). In any event, it is significant that acidification was also demonstrated by using an endosome fraction from BHK-21 fibroblasts that is not only devoid of lysosomal hydrolases (11) but can also be labeled under conditions (temperatures <20°C) in which endosome–lysosome fusion is inhibited (11, 21).

Although defining the mechanism of endosome acidification will require further study, some initial conclusions can be reached. The inhibitor studies (Fig. 4) clearly indicate that the endosome H^+-ATPase is distinct from the F_1,F_0-type enzyme mitochondria, chloroplasts, and bacteria (which are efrapeptin-, NaN_3-, and DCCD-sensitive) and from the class of phosphoenzyme ion-pumping ATPases, including Na^+,K^+-ATPase, Ca^{2+}-ATPase, and K^+,H^+-ATPase (which are vanadate-sensitive) (22, 23). Of the agents tested, only *N*-ethylmaleimide inhibited the ATP-driven acidification of endosomes. Because these results are virtually identical to those obtained for the lysosome fraction—as well as for purified rat liver lysosomes (13)—it is possible that the same H^+-ATPase (or class of H^+-ATPase) acidifies both endosomes and lysosomes. Further, this enzyme may be identical to H^+-ATPases that acidify many nonendocytic organelles, including secretory granules and storage vacuoles (22). In secretory granules, this enzyme is known to be electrogenic (24–26). However, we have noted that low concentrations of FCCP fail to prevent acidification in the absence of valinomycin (data not shown), which may suggest that part of the H^+ pumping is compensated by counter ion movement.

Despite the similarity between endosomes and lysosomes, some differences were apparent (Figs. 2 and 3 *C* and *E*). These differences could be explained if lysosomes contain more internal buffering capacity and consequently are more acidic as isolated. Accordingly, their internal pH would rise gradually in KCl buffer and would require a greater H^+ influx to overcome the internal buffering. Thus, removal of permeant ions inhibits lysosome acidification more than that of endosomes and similarly decreases the ability of FCCP alone to reverse any H^+ gradient so established (Fig. 3 *B*, *C*, and *E*).

Considerable attention has been paid recently to the possible functions of a prelysosomal acidic compartment (1, 2). Although its role is incompletely understood, it is clear that several types of receptor–ligand complexes should dissociate upon reaching the low pH of the endosome. Thus, free receptors would be generated to return to the plasma membrane without exposure to the acidic, but proteolytically dangerous, environment of the lysosome.

How are H^+ pumps inserted into the endosome membrane? If the H^+-ATPase is a constituent of a cell's plasma membrane, it might be interiorized during pinocytosis—as are many other plasma membrane proteins (14). This concept has received initial support from the recent work of Forgac *et al.* (27), who have demonstrated the ATP-dependent acidification of isolated coated vesicles, presumably the primary pinocytic compartment (1, 2). This coated-vesicle H^+-ATPase has similar properties to the endosome/lysosome ATPases described here.

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